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T-2 Toxicosis and Blood Coagulation in Young Chickens[1,2,3]

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T-2 Toxicosis and Blood Coagulation in Young Chickens. DOERR, J. A., HAMILTON, P. B., AND BURMEISTER, H. R. (1981). *Toxicol. Appl. Pharmacol.* **60,** 157–162. Recent reports have suggested that T-2 toxicosis may be a contributing factor in hemorrhagic manifestations such as alimentary toxic aleukia in man and hemorrhagic anemia syndrome and general clotting dysfunction in animals. The present communication describes the effect of graded levels of dietary T-2 toxin (0, 1, 2, 4, 8, and 16 μg/g) on the activities of tissue thromboplastin, coagulation Factors VII, X, and V, prothrombin, and fibrinogen in young chickens. In the extrinsic clotting pathway a qualitative change was demonstrated in the lipid fraction of thromboplastin, and Factor VII activity was significantly ($p > 0.05$) reduced by the growth inhibitory dose of 4 μg/g. Of the common pathway functions, Factor X, prothrombin, and fibrinogen were depressed by 16 μg/g T-2 toxin, with the latter two parameters being reduced to below 60% of normal. Factor V was unaffected. These data show that T-2 toxin can produce a distinct coagulopathy in chickens characterized by a primary defect in Factor VII activity and secondary effects on prothrombin and fibrinogen. Additionally, the effects of T-2 toxicosis on clotting competence provide further evidence of a possible role of T-2 toxin in alimentary toxic aleukia.

T-2 toxin is a mycotoxin produced by several species of the genus *Fusarium* (Bamberg *et al.*, 1970) that grow in cereals, feeds, and vegetables. A naturally occurring sesquiterpenoid, its chemical name is 4,15-diacetoxy-8-(3-methyl-butyryloxy)-12,13-epoxy-Δ^9-trichothecen-3-ol (Bamberg *et al.*, 1968). T-2 toxicosis as a field problem was discovered when cattle succumbed to hemorrhagic disease caused by ingestion of moldy feed, and analysis of suspect feed showed T-2 toxin present at 2 μg/g (Hsu *et al.*, 1972). Pathre and Mirocha (1977) reported that T-2 toxin caused bloody stools in cattle. T-2 toxin has been implicated in alimentary toxic aleukia (ATA) in man (Wyatt *et al.*, 1972), which includes hemorrhagic episodes among its symptoms (Mayer, 1953). The fungal producers of T-2 toxin have been implicated in the hemorrhagic anemia syndrome (HAS) of poultry described by Forgacs and Carll (1962). Additionally, Doerr *et al.* (1974) reported that prothrombin times of chickens were elevated during T-2 toxicosis.

These reports linking T-2 toxin to bleeding events prompted the current investigation of the specific sites in the coagulation mechanism affected by this important mycotoxin.

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The present experiments in chickens were designed to elucidate both the nature and the extent of the lesions resulting in coagulopathy, to further the descriptive knowledge of avian T-2 toxicosis, and perhaps to gain further insight into ATA and HAS.

METHODS

Day-old male broiler chickens (Pilch × Pilch) were obtained from the University farm and were housed under continuous illumination in electrically heated batteries with wire floors. Feed and water were available *ad libitum*. The diets were fed for 21 days, at which time the experiments were terminated.

T-2 toxin was produced by growing *Fusarium tricinctum* strain NRRL 3299 on white corn grits, followed by extraction and purification of the toxin by the method of Burmeister (1971). The procedure yielded a crystalline product having a melting range of 148 to 150°C. T-2 toxicosis was induced by adding known amounts of crystalline toxin to a commercial broiler-starter feed that was free of any medication. The toxin was dissolved in 50% (v/v) aqueous ethanol and added to small portions of the feed. After drying at 100°C, these portions were incorporated into the remainder of the feed. Dose levels of 0, 1, 2, 4, 8, and 16 μg of T-2 toxin/g of diet were thus obtained.

A completely randomized experimental design was used. There were four replicates of 10 birds at each dose level. Replicate means were evaluated by analysis of variance, and the treatment means were compared by the method of least significant differences. Data reported as percentages were submitted to logarithmic transformation prior to analysis of variance (Snedecor and Cochran, 1967).

Thromboplastin for determination of activity of coagulation factors was prepared from acetone-dehydrated brain powder from control chickens according to the method of Griminger *et al.* (1970) with the modifications of Doerr *et al.* (1975). The effect of toxin on tissue thromboplastin was determined by diluting to extinction through twofold serial dilutions the brain powder prepared from affected birds.

Blood was collected by cardiac puncture, and plasma was prepared by the method of Doerr *et al.* (1975). Factor analyses were performed as described by Doerr *et al.* (1976) using commercially obtained substrate plasmas[5] deficient in either Factor V, Factor VII, or Factor X. Prothrombin was measured by the method of Owren as described by Simmons (1968). Fibrinogen was measured according to Goodwin as described by Frankel and Reitman (1963). Thromboplastin protein was measured by the biuret method (Wooton, 1964), and lipid by the gravimetric method of Friedman (1968). *In vitro* clot retraction was measured by the modified Tocantins technique described by Simmons (1968) except that frilled, cellophane-wrapped cocktail picks[6] were substituted for wooden applicator sticks to facilitate the withdrawal of the clots.

RESULTS

Tissue thromboplastin is the initiating activator of the extrinsic blood coagulation pathway in mammals and chickens (Doerr *et al.*, 1975). The activity of thromboplastin from healthy birds was compared with that obtained from birds suffering severe (16 μg/g) T-2 toxicosis. The activity curves of the control birds and intoxicated birds (Fig. 1) after twofold serial dilutions of tissue thromboplastin had the same general shape, with an initial constant activity through three dilutions and a mean prothrombin time of 10.5 sec. This was followed in both cases by a smooth but rapid increase in prothrombin time through dilutions 4 to 13, indicating a loss of thromboplastin activity on dilution. The thromboplastin activity in this early portion of the curve is protein dependent (Doerr *et al.*, 1976). At dilutions 14 and 15, the activity curves of both thromboplastin preparations reached plateaus in which the prothrombin times of the experimental preparations were significantly ($p > 0.05$) increased over the control values. Because the activity in the plateau region is attributed to the lipid fraction of thromboplastin (Doerr *et al.*, 1976), the prolonged prothrombin times associated with T-2 toxicosis suggested a decrease in the lipid fraction. The total lipids were measured and found to be unaltered during T-2 toxicosis. The plateau region was followed by a second rapid loss of activity until the recalcification times of the tested plasmas were reached.

[5] Supplied by DADE Division, American Hospital Supply Corporation, Miami, Fl. 33152.

[6] Supplied by Party Frills, American Roland Food Corporation, New York.

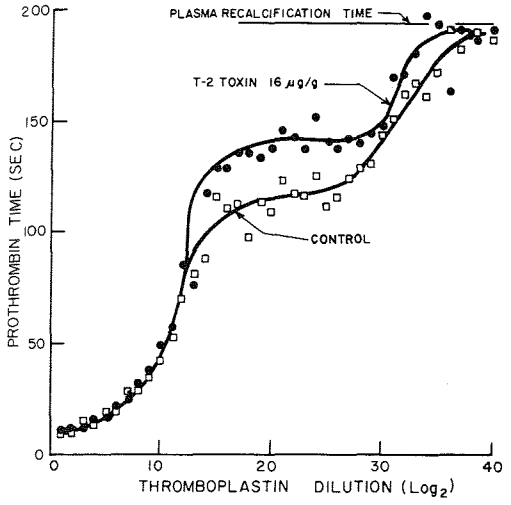

FIG. 1. Effect of dietary T-2 toxin on brain thromboplastin activity.

The activities of extrinsic/common pathway clotting factors during T-2 toxicosis are shown in Fig. 2. In the coagulation scheme (Sirridge, 1974), which we adopted as a model for analysis of clotting competence, Factor VII (known also as proconvertin or stable factor) is activated by tissue thromboplastin and then combines with thromboplastin constituents to form an enzymatically active complex, which is required for the activation of Factor X. In our assays, Factor VII activity was depressed significantly ($p < 0.05$) by T-2 toxin at doses of 4 μg/g and above. At the highest dose, Factor VII was depressed by 38%.

Factor X (Stuart factor) occupies a critical step in the clotting process in that it forms a juncture for both the intrinsic and the extrinsic clotting pathways. It is the primary enzymatic constituent of the prothrombin converting complex formed by either coagulation route. T-2 toxin produced a slight but significant ($p < 0.05$) depression of Factor X activity only at the highest dose of toxin tried (16 μg/g).

Factor V (accelerator globulin or labile factor) serves as a cofactor in that, in conjunction with activated Factor X (prothrombinase), a calcium ion, and phospholipid, it accelerates the conversion of prothrombin to thrombin. The slight reduction of Factor V activity by T-2 toxin was statistically insignificant ($p < 0.05$).

Prothrombin, the circulating immediate precursor of thrombin, can be considered the final enzyme of the hemostatic cascade because it catalyzes the hydrolytic cleavage of fibrinogen to monomeric fibrin, which is the culminating constituent of a blood clot. Prothrombin activity was diminished significantly ($p < 0.05$) only by the highest dose of T-2 toxin. The reduction was to 55% of the corresponding control.

Fibrinogen, the circulating substrate for the clotting pathway common to both extrinsically and intrinsically initiated coagulation, was reduced in concentration from 305 to 176 mg/100 ml in birds fed 16 μg/g. Lower dietary levels of T-2 toxin had no significant ($p < 0.05$) effect on fibrinogen (Table 1).

The *in vitro* retraction of clots is the phenomenon permitting the collection of serum from whole blood; this retraction, which is altered during aflatoxicosis (Doerr *et al.*, 1976), is sensitive to at least three variables, namely, fibrinogen level, platelet

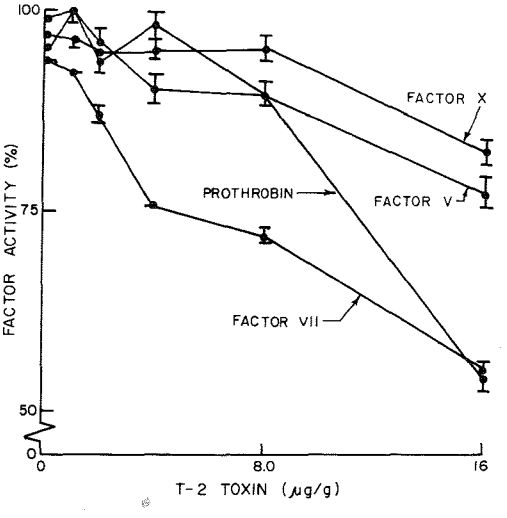

FIG. 2. Effect of graded levels of dietary T-2 toxin on clotting factor activities. Each point represents the mean of four groups of 10 birds; vertical bars are SE.

TABLE 1

EFFECT OF T-2 TOXIN ON PLASMA FIBRINOGEN AND CLOT RETRACTION IN YOUNG BROILER CHICKENS

T-2 toxin (μg/g)	Fibrinogen (mg/100 ml)	Clot retraction (%)
0	305 ± 16[a]	61.9 ± 0.9[a]
1.0	279 ± 13	60.1 ± 0.3
2.0	254 ± 13	64.5 ± 2.0
4.0	263 ± 24	60.9 ± 1.5
8.0	267 ± 19	60.0 ± 1.8
16.0	176 ± 20*	68.5 ± 2.1

[a] Tabular values are the means of 40 birds ± SE.
* This value differed significantly ($p < 0.05$) from the control value.

(thrombocyte) numbers, and the activity of a retractile protein known as thrombosthenin (Deykin, 1973). Because T-2 toxin decreased the fibrinogen level, clot retraction (Table 1) was measured and found to be increased slightly but not significantly ($p < 0.05$).

DISCUSSION

The current investigation was based on the assumption that the mechanism of avian blood coagulation resembles in broad aspects the extrinsic and common clotting pathways described in humans, an assumption which is supported in the literature (Griminger, 1965; Archer, 1971; Stopforth, 1970) and which proved useful in evaluating and describing the coagulopathy of aflatoxicosis (Doerr *et al.*, 1976). Because it is generally accepted that a loss of about 70% of a single clotting factor is required to prolong prothrombin times in humans (Sirridge, 1974), the increased prothrombin times reported during T-2 toxicosis (Doerr *et al.*, 1974) suggested a marked alteration in coagulation during this mycotoxicosis. The results of the current investigation support this contention.

Analysis of the individual factors of the enzymatic cascade responsible for the extrinsic/common pathway of coagulation revealed that Factor V was refractory, whereas Factor VII, Factor X, prothrombin, and fibrinogen were reduced in concentration during severe T-2 toxicosis (Fig. 3). Factor X, prothrombin, and fibrinogen were lowered only at the highest level (16 μg/g) of dietary T-2 toxin. Factor VII, being affected at 4, 8, and 16 μg/g, was the most sensitive clotting component. Thus, severe T-2 toxicosis emerges as a multiple-site coagulopathy, whereas milder T-2 toxicosis would be essentially a specific deficiency of Factor VII. This contrasts with aflatoxicosis, which also is a multiple-site coagulopathy but is primarily a hypoprothrombinemia (Doerr *et al.*, 1976).

T-2 toxin has been reported to act as an inhibitor of initiation but not of elongation and termination of protein synthesis on ribosomes in eukaryotic cells (McLaughlin *et al.*, 1977), and presumably the decrease in circulating clotting factors during T-2 toxicosis is a consequence of this. How-

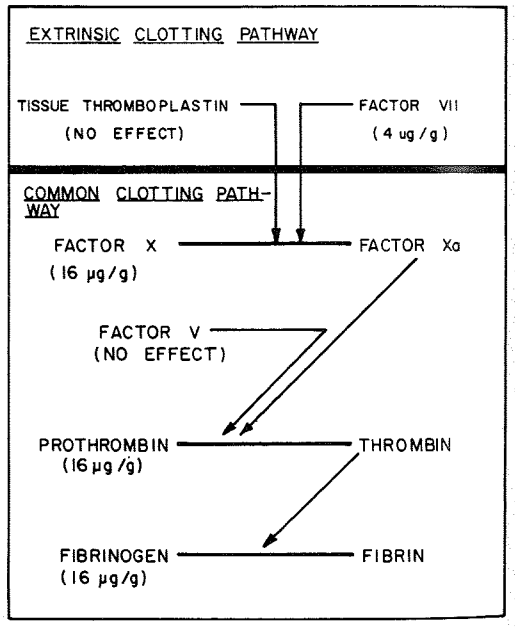

FIG. 3. Extrinsic/common clotting pathway; the numbers in parentheses are the minimum doses of T-2 toxin required to cause significant ($p < 0.05$) effects.

ever, there must be some specificities in the mechanism because Wyatt *et al.* (1973) detected no significant effect on total serum proteins during T-2 toxicosis in chickens, and the current results showed a specific effect on Factor VII. Perhaps it is pertinent that the circulating clotting factors are synthesized in the liver, indicating that at least a limited hepatotoxicity is associated with T-2 toxin, probably at the subcellular rather than the cellular level.

The alteration in thromboplastin activity during T-2 toxicosis is intriguing. The alteration was detectable only at high dilutions (Fig. 1), and the difference from control values did not appear of great consequence (about 20% in prothrombin times). However, the alteration was associated with the second or lipoidal component in the thromboplastin dilution curve even though the total lipids of thromboplastin were unaltered. This latter observation agrees with those of Wyatt *et al.* (1973) that serum total lipids, serum cholesterol, and liver lipids are not altered during T-2 toxicosis. A likely explanation for the alteration associated with the lipoidal component would be a qualitative shift in thromboplastin composition. Such changes in composition have been reported to alter blood clotting (Hecht, 1965).

The role of T-2 toxin in hemorrhagic events associated with diseases such as ATA and HAS remains somewhat uncertain. T-2 toxin unquestionably can cause a reduction in blood clotting factors in chickens; although a level of 16 μg/g was required to cause a multiple-site coagulopathy, only 4 μg/g is required to inhibit growth in this experimental system (Wyatt *et al.*, 1973). A Factor VII deficiency of about 25% occurred at the minimal growth inhibitory dose of 4 μg/g, and prothrombin times are reduced only at 8 and 16 μg/g (Doerr *et al.*, 1974). These facts suggest that interference with blood clotting is not a major effect or symptom of T-2 toxicosis, particularly since dietary levels as low as 0.5 μg/g can cause oral lesions in this experimental system (Wyatt *et al.*, 1972). Nevertheless, comparison of the present results with ATA or HAS must take into account that ATA and HAS are field outbreaks where the potential for interactions with other mycotoxins and environmental factors are well documented (Lillehoj and Ciegler, 1975; Hamilton and Harris, 1971), whereas the current studies used animals maintained in a chronic, steady-state condition. Considered altogether, these items suggest that although T-2 toxin will produce hemorrhagic problems, the problems will be accompanied by other manifestations.

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